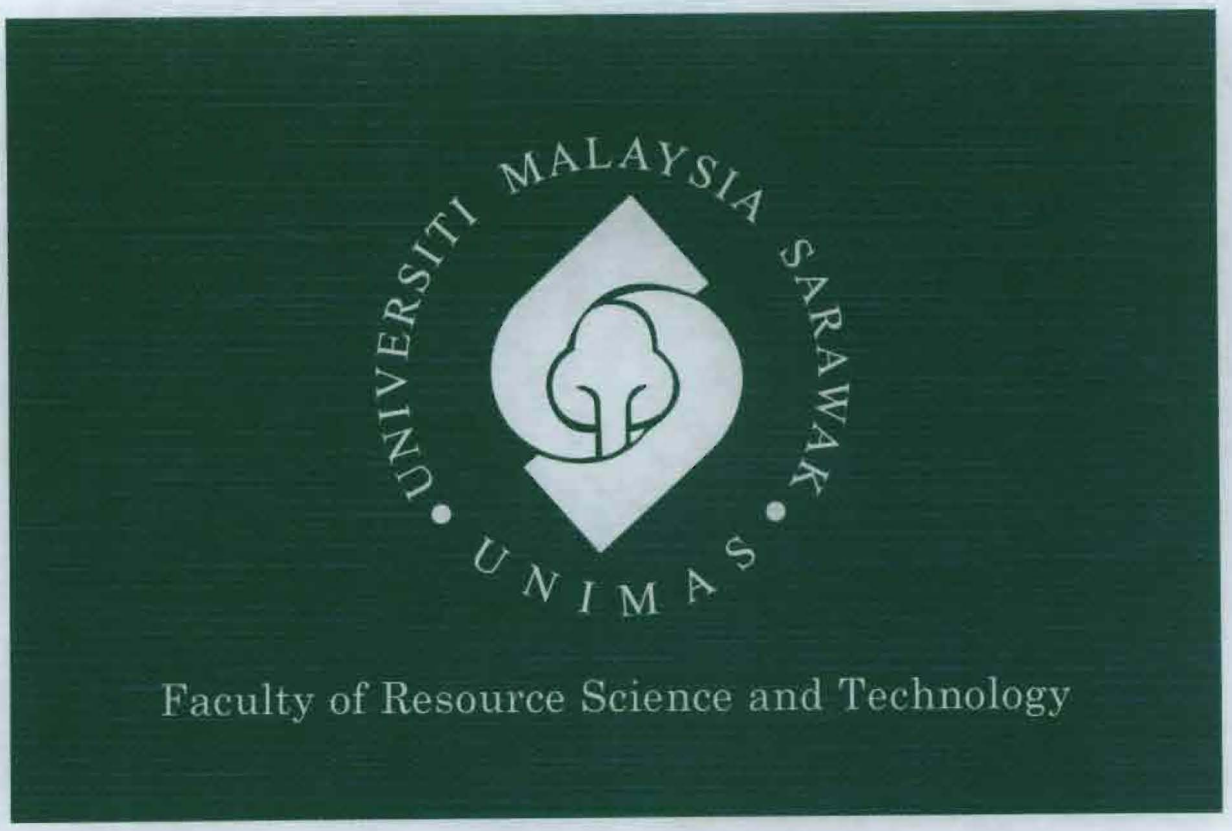

ISOLATION OF GENOMIC CLONES OF UDP-N-acetylglucosamine pyrophosphorylase (AGX) FROM KELAMPAYAN (*Neolamarckia Cadamba*)

Tiong Yong Wei

Bachelor of Science with Honours
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Isolation of Genomic Clones of UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) from Kelampayan (*Neolamarckia cadamba*)

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours

Resource Biotechnology
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DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

AGM1	Phospho-N-acetylglucosamine mutase
AGX	UDP-N-acetylglucosamine pyrophosphorylase
Bp	Base pair (s)
cDNA	Complementary DNA
CIA	Chloroform:isoamyl alcohol
CTAB	Cetyltrimethylammonium bromide
ddH_2O	Distilled deionized water
DBH	Diameter at breast height
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EST	Expressed Sequence Tag
Fructose-6-P	Fructose-6-phosphate
GFA1	Glutamine-fructose-6-phosphate amidotransferase
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GlcNAc-1-P	N-acetylglucosamine-1-phosphate
Glucosamine-6-P	Glucosamine-6-phosphate
Glucose-6-P	Glucose-6-phosphate
GNA1	Glucosamine-6- phosphate N-acetyltransferase
GPI	Glycosylphosphatidylinositol
GPS	Global Positioning System
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction

PPi	Pyrophosphate
PVP	Polyvinylpyrrolidone
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
UDPGP	Uridine diphosphate glucose pyrophosphorylase
UDP-sugar	Uridine diphosphate-sugar
UTP	Uridine triphosphate

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Isolation of Genomic Clones of UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) from Kelampayan (*Neolamarckia cadamba*)

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ABSTRACT

Neolamarckia cadamba or locally known as Kelampayan is an evergreen and tropical tree native to South Asia. Kelampayan possesses great economic and commercial value as its timber is the best raw materials for plywood industry. UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) gene is a key enzyme used for the cell wall formation in chitin synthesis pathway. The presence of chitin helps in providing tensile strength of the cell wall that is required to maintain turgor and compensation mechanism activated by cell wall damage. In this study, the isolation of genomic clones of *AGX* gene from *N. cadamba* was studied. The main objective was to isolate the genomic clones of *AGX* gene from *N. cadamba*. The targeted DNA sequence of *AGX* gene was amplified with the designed primer pair based on EST sequence which was obtained from EST of *Neolamarckia cadamba* (NcdbEST) by using Polymerase Chain Reaction (PCR) technique. The genomic sequences of *AGX* gene were subjected to nucleotide BLAST (BLASTn) analysis to search for homology sequence and validate the identity of the obtained sequences through NCBI. Unfortunately, the *AGX* genomic sequences did not show similarity to *AGX* gene but having similarity towards *Aquaporin* gene. Therefore, the desired primers obtained from NcdbEST need to be redesigned for other researches that involved in using *AGX* gene in future.

Key words: *Neolamarckia cadamba*, EST of *Neolamarckia cadamba* (NcdbEST), polymerase chain reaction (PCR), UDP-N-acetylglucosamine pyrophosphorylase (*AGX*), Nucleotide BLAST (BLASTn)

ABSTRAK

Neolamarckia cadamba atau lebih dikenali sebagai Kelampayan merupakan suatu malar hijau dan pokok tropika asli yang boleh dijumpai di Asia Selatan. Kelampayan mempunyai nilai ekonomi dan komersial yang tinggi iaitu sebagai kayu untuk bahan-bahan mentah terbaik bagi industri papan lapis. Gen UDP-N-acetylglucosamine pyrophosphorylase (AGX) merupakan suatu enzim utama yang digunakan dalam sintesis chitin laluan. Kehadiran chitin membantu dalam menyediakan kekuatan tegangan dinding sel yang diperlukan untuk mengekalkan turgor dan pampasan mekanisme yang diaktifkan oleh kerosakan dinding sel. Dalam kajian ini, pengasingan klon genomik AGX daripada N. cadamba telah dikaji. Objektif utama dalam kajian ini adalah untuk mengasingkan klon genomik gen AGX dari N. cadamba. Urutan DNA sasaran gen AGX telah dikuatkan dengan pasangan pencetus direka berdasarkan urutan EST yang diperolehi daripada EST daripada Neolamarckia cadamba (NcdbEST) dengan menggunakan teknik tindakbalas berantai polimerase (PCR). Urutan genomik AGX adalah tertakluk kepada analisis BLAST nukleotida (BLASTn) untuk mencari urutan homologi dan mengesahkan identiti urutan diperolehi melalui NCBI. Malangnya, urutan genomik AGX tidak menunjukkan persamaan dengan gen AGX tetapi terdapat persamaan dengan gen Aquaporin. Oleh itu, pencetus yang diperolehi daripada NcdbEST perlu direka semula untuk kajian lain yang terlibat dalam menggunakan gen AGX pada masa depan.

Kata kunci: *Neolamarckia cadamba, EST daripada Neolamarckia cadamba (NcdbEST), tindakbalas berantai polimerase (PCR), gen UDP-N-acetylglucosamine pyrophosphorylase (AGX), BLAST nukleotida (BLASTn)*

1.0 INTRODUCTION

Neolamarckia cadamba or locally known as Kelampayan is a large, deciduous and fast growing tree species with short rotation cycle for planted forest development in Sarawak that gives early economic returns within 6-8 years. It is known as a "miracle tree" in China due to its fast growing characteristics and is also an ideal tree species to study genetic functions related to tree growth and cell wall development (Li *et al.*, 2011). Kelampayan is an indigenous species for areas from India, Nepal, through Thailand and Indo-China and eastward in the Malaysian Archipelago to Papua New Guinea (Joker, 2000). Its habitat can be found in freshwater swamps and logging area of lowland dipterocarp forests (Nair, 2007). It is the best material for plywood industry and raw materials for pulp and paper industry. Thus, Kelampayan is favoured in plantation programs due to its multipurpose function and utility.

Recently, the global demand for wood products increases which leads to an increase in forest productivity. Hence, it is inevitable to come up with strategy in order to cater the current demand of the improved planting materials by producing more comprehensive tree improvement programme with a long-term strategy in producing genetically improved planting materials. The Sarawak Government has introduced the Forest (Planted Forest) Rules (1997) to encourage the development of commercial forest plantations by setting a target of one million hectares of planted forests by the year 2020. Hence, the Sarawak Government has identified Kelampayan as one of the priority species for large-scale tree plantations as it plays a significant role in the economic sector. By achieving this target, there is a need to invest more in the research and development (R&D) of high-yielding, faster growth and short-rotation planted forests to increase the competitiveness of the forest-based sector in Sarawak.

Cell wall damage is caused by mutations of cell-wall-related genes that trigger a compensatory mechanism which eventually results in hyperaccumulation of chitin (Lagorce *et al.*, 2002). Chitin is a long chain polymer of N-acetylglucosamine and is a derivative of glucose. Walls of evolutionary advanced fungi and plants possess multi-layered microfibrillar networks of chitin or cellulose (Caprita, 1985). The presence of chitin helps in providing tensile strength of the cell wall that is required to maintain turgor and compensation mechanism activated by cell wall damage (Lagorce *et al.*, 2002). UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) is one of the plant pyrophosphorylases in producing UDP-sugars which is the key precursors for glycosylation reaction and acts as a key enzyme in cell wall formation of chitin synthesis pathway (Szumilo *et al.*, 1996). Therefore, extensive study needs to be carried out in order to determine the genomic clones of the *AGX* quality in chitin synthesis gene of the cell wall on Kelampayan plant species that were selected randomly from the wild stands in Kota Samarahan, Sarawak.

Hence, the primary goal of this study was to isolate the genomic clones of UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) genomic sequence from Kelampayan plant species.

2.0 LITERATURE REVIEW

2.1 *Neolamarckia cadamba* (Kelampayan)

Neolamarckia cadamba or locally known as Kelampayan is a member of the tribe Neolamarckia in the family of Rubiaceae. *N. cadamba* is a medium to tall growing deciduous tree that attained a height of 45 m and 2.4 m in girths, with a clear bole of 9 m (Mondal *et al.*, 2011). The diameter of *N. cadamba* can be up to 100 cm as well as the crown is umbrella-shaped and the branches are characteristically arranged in tiers (Joker, 2000). It is typical pioneer and commonly found in secondary forest. It is widely dispersed in lowland to mountain forests of up to 1000 m altitude where there is more than 1500 mm rain/year. However, it can also grow in dry areas with as little as 200 mm rain/year. *N. cadamba* can grow on a variety of soils and tolerates periodic flooding (Joker, 2000).

According to Patel and Kumar (2008), the shape of *N. cadamba* leaves is broadly ovate, elliptic-oblong with entire margin and pulvinus base. It is bitter in taste and its length of the leaves varies from 7.5 to 18 cm and breath is 4.5 to 16 cm. The leaf is dorsiventral with thick prominent midrib and uniformly thin lamina in the microscopic studies. Furthermore, the powder form of the leaf showed the presence of unicellular, lignified trichomes, paracytic stomata, simple starch grains and sandy balls of calcium oxalate crystals through the microscopic studies.

N. cadamba is categorized as an industrial species as it produces one of the best sources of raw materials for plywood industry, besides pulp and paper production. Due to the timber has light and soft properties, it promotes the usage for light-weight purposes such as molding, skirting, disposable chopsticks, wooden sandals, match splinters, packing cases, panel boards, picture frames, pencils and various other products (Lim *et al.*, 2005).

According to Divyakant *et al.* (2012), various parts of the *N. cadamba* plant have traditional uses. The barks and leaves of the plant are reported to have good medicinal values in traditional system of medicines such as astringent, anti-hepatotoxic, anti-diuretic, wound healing, antiseptic and anthelmintic. Furthermore, the dried bark can be used to relieve fever and as a tonic, whereas a leaf extract can be served as a mouth wash (Mondal *et al.*, 2011). Additionally, the seeds have great medicinal value as it cures astringent, ulcer, fever, vomiting and diarrhea (Peter, 2007).

(a) **(b)**

Figure 2.1 *Neolamarckia cadamba.* (a) The flower and leaves of *N. cadamba*, and (b) Tree trunk with coarse surface of *N. cadamba.*
(Adapted from (a) http://farm6.staticflickr.com/5219/5384490304_d605ec4770.jpg (b) http://farm3.staticflickr.com/2123/2078207818_7806dae7e3_z.jpg)

2.2 Wood Formation

According to Plomion *et al.* (2001), wood is classified as the fifth most important product of the world trade. It is an environmentally acceptable future alternative to fossil fuel resources by providing fuel, fibres and sawn timber as commodities. The chemical composition of wood such as cellulose, hemicellulose, lignin and pectins makes it an ideal raw material for future "ligno-chemical" industry that could replace the petrochemical

industry. Moreover, developing wood represents one of the most important sink for the removal of excess atmospheric carbon dioxide, thereby alleviating some of the dangers attributable to global warming (Chaffey *et al.*, 2002).

The development of wood to a final heartwood stage involves several processes such as cambial division, wood-cell expansion, wood-cell maturation, sapwood formation and heartwood formation (Bailey, 1952). According to Zhang *et al.* (2011), wood or also known as secondary xylem, consists of xylem parenchyma cells and two types of elongated water-conducting cells with thickened secondary cell wall, tracheary elements and fibres. The vascular development in plants is divided into two phases which are primary and secondary development. During primary development in the stem and root, the conductive tissue (xylem and phloem) are formed from the procambial tissue associated with the apical meristems. Vascular cambium is established through further asymmetric periclinal cell divisions of procambial cells and thus it is dedicated to secondary vascular development. The cambial cells start to proliferate and give rise to secondary phloem outwards and secondary xylem inwards.

According to Demura and Fukuda (2006), from Figure 2.2 (a), procambial cells and daughter cells are produced by cambial initials and thus they are differentiating into phloem cells and xylem (wood) cells. Xylem (wood) cells include tracheary elements and fibres whereas tracheids and vessels are constituents of tracheary elements. There are two types of vessels observed in angiosperms which are the protoxylem vessels and metaxylem vessels. Protoxylem vessels commonly have annular and spiral secondary wall thickenings whereas metaxylem vessels usually have reticulate and pitted thickenings.

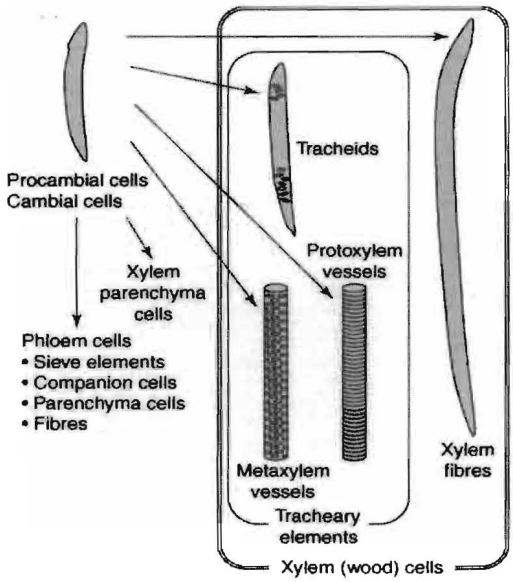

Figure 2.2 (a) Schematic model of wood formation. (Adapted from Demura, T. & Fukuda, H. (2006). Transcriptional regulation in wood formation. *TRENDS in Plant Science*, 12 (2), 64-70.)

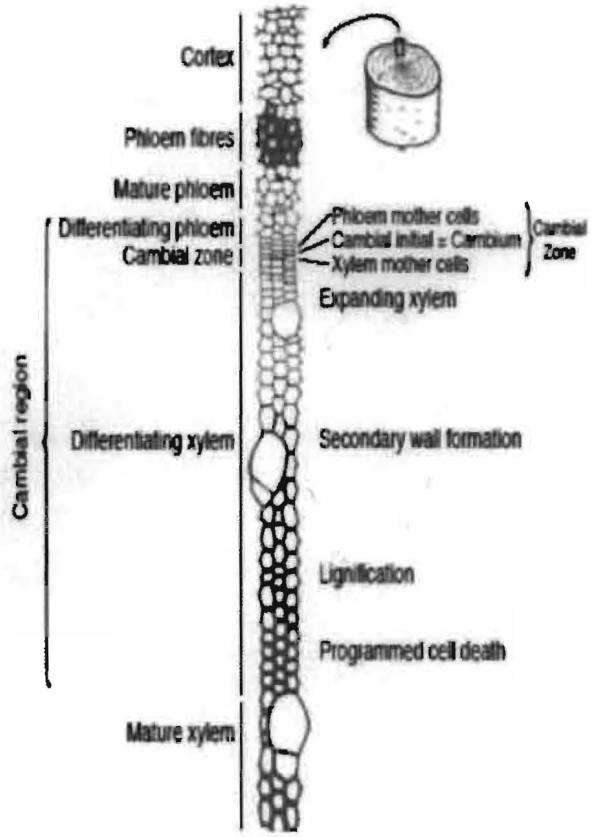

Figure 2.2 (b) Wood formation process. (Adapted from http://fgilab.com/wp-content/uploads/2010/05/biotechcol3_wsho.pdf).

Xylem development or known as wood formation occurs through continuous steps involving cambium cell proliferation, xylem cell specification and expansion, secondary cell wall deposition and programmed cell death to form hollow tube-like cells (Zhang *et al.*, 2011). Derivative cell expand longitudinally and radically during the primary cell wall formation. Primary determinants involved in this process are xyloglucan endotransglycosylases, endoglucanases, expansins, pectin methyl esterases and pectinases (Plomion *et al.*, 2001). Formation of secondary cell wall is driven by the coordinated expression of numerous genes specifically involved in the biosynthesis of cell wall proteins and polysaccharides (cellulose and hemicelluloses) and formation of lignin. Finally, hollow tube-like cells is formed with secondary walls when programmed cell death happens (Demura and Fukuda, 2006).

2.3 Chitin Synthesis Pathway

Chitin is a polymer of N-acetylglucosamine and is an important component of fungal pathogenicity. Chitin is mainly found in fungal cell walls and minority in plant cell walls which only accounts 1±3 % of the cell wall dry mass (Orlean, 1997). The major part of chitin is localized as a ring at the base of the bud. A small part of the chitin is deposited in dispersed fashion in the lateral walls and it is covalently linked to β-1,3-glucans and β-1,6-glucans (Kollar *et al.*, 1995). Mutations do occurs in genes that involved in the biosynthesis of β-1,3-glucans, mannans or cell-wall component assembly, accompanied by dramatic changes in the molecular morphology of the cell wall (Smits *et al.*, 1999). These results in a large increase in chitin formation as it can rise to 20 % of the cell wall dry mass. Hence, the activation of chitin synthesis contributes to the strength of the cell wall as well as compensation mechanism activated by cell wall damage (Lagorce *et al.*, 2002).

According to Wan *et al.* (2008), although plants lack chitin, they do secrete chitin-degrading enzymes. Plant cells secrete chitin-degrading enzymes that release chitin fragments (chitooligosaccharides or chitin oligomers) during fungal infection that acts to induce plant innate immunity against the invading pathogen. Overexpression of chitin-degrading enzymes led to enhanced resistance to fungal pathogens. Furthermore, pretreatment of plants with chitooligosaccharides enhances plant resistance against various pathogens. Additionally, recent gene expression profiling studies demonstrated that chitooligosaccharides were a potent regulator of plant gene expression. Hence, the presence of chitin-degrading enzymes in plants mediates plant disease resistance.

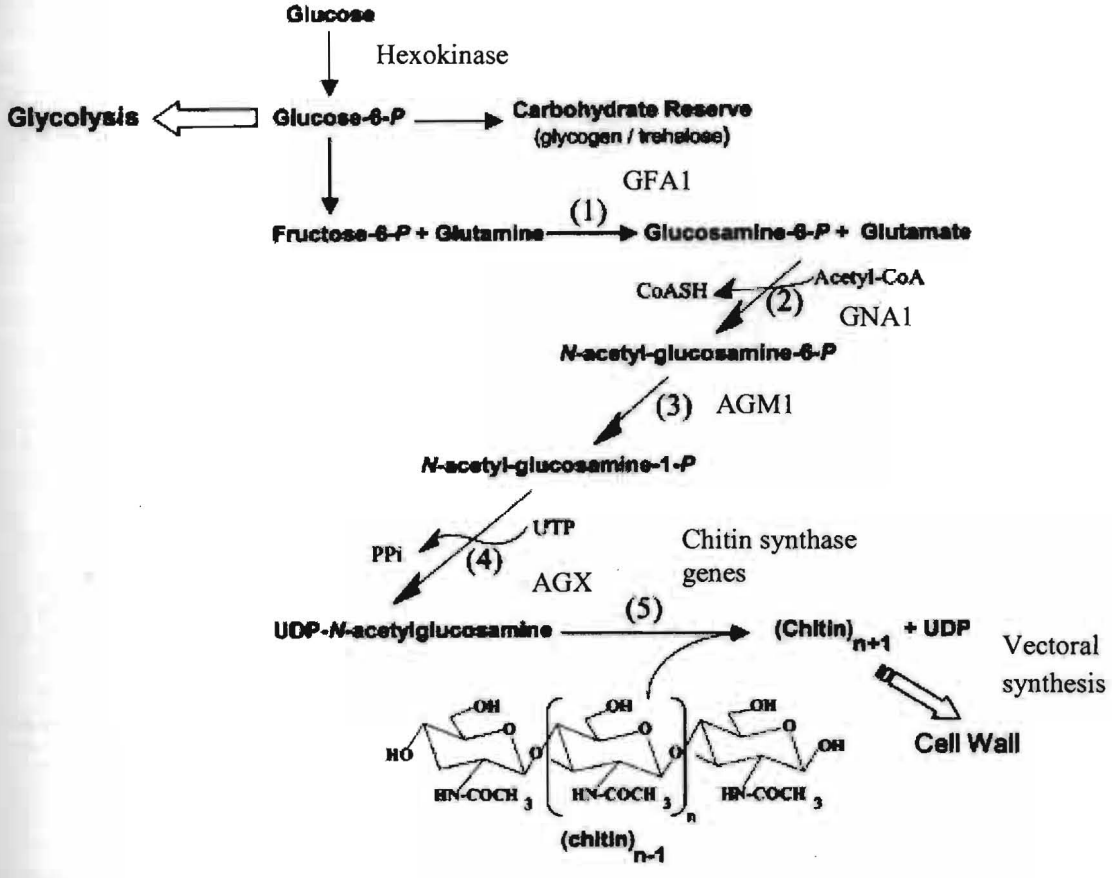

Figure 2.3 Interaction of the chitin synthesis pathway with glycolysis and cell-wall synthesis. (Adapted from Lagorce, A., Berre-Anton, V.L., Aguilar-Uscanga, B., Martin-Yken, H., Dagkessamanskaia, A., & Francois, J. (2002). Involvement of GFA1, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*. *European Journal of Biochemistry*, 269, 1697-1707.)

According to the study conducted by Lagorce *et al.* (2002), from Figure 2.3, glucose enters the cell via a glucose transporter and being converted to glucose-6-phosphate (glucose-6-P) by hexokinase. Then, there are two main processes which are the glycolysis pathway and converted to fructose-6-phosphate (fructose-6-P) pathway. The combination of fructose-6-P and glutamine converted to form glucosamine-6-phosphate (glucosamine-6-P), that glutamine serves as the donor of the amino group. This reaction is catalysed by the rate-limiting enzyme glutamine-fructose-6-phosphate amidotransferase (GFA1). Glucosamine-6-P is rapidly acetylated through the action of acetyl-CoA to form N-acetylglucosamine-6-phosphate (N-acetylglucosamine-6-P) by glucosamine-6-phosphate N-acetyltransferase (GNA1). After that, N-acetylglucosamine-6-P is isomerized to N-acetylglucosamine-1-phosphate (GlcNAc-1-P) by phospho-N-acetylglucosamine mutase (AGM1) and activated, via the action of UDP-N-acetylglucosamine pyrophosphorylase (*AGX*), to UDP-N-acetylglucosamine (UDP-GlcNAc) that serves for the synthesis of chitin, glycoproteins and glycosylphosphatidylinositol (GPI) anchors of some membrane proteins as well as for the modification of other substrates (Arakane *et al.*, 2011). The synthesis of chitin is mediated by chitin synthase genes which is an integral membrane enzyme that catalyses the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a growing chitin chain.

According to Bowman and Free (2006), the elongation of the chitin polymers occurs through vectoral synthesis. The newly formed polymers of chitin contains the hydrogen bonding which results in microfibril formation and subsequent crystallization of chitin in the extracellular space which is adjacent to the plasma membrane. This process of chitin synthesis primarily occurs at sites of active growth and involves the assembly, cross-

linking and remodeling of cell wall. Thus, the chitin provides cell wall with mechanical strength and integrity as well as compensation mechanism activated by cell wall damage.

2.4 UDP-N-acetylglucosamine pyrophosphorylase (*AGX*)

UDP-N-acetylglucosamine pyrophosphorylase (*AGX*) is one of the plant pyrophosphorylases that are capable of producing UDP-sugars which is the key precursors for glycosylation reactions (Szumilo *et al.*, 1996). *AGX* is an important enzyme in eukaryotic cells especially for the component of the cell walls of yeast and fungi as well as a precursor of the peptidoglycan of many prokaryotic cells (Ballou, 1982). Furthermore, *AGX* is responsible for the formation of UDP-GlcNAc, which serves as a donor nucleotide sugar and is an essential precursor for the GlcNAc residues in N-linked oligosaccharides, proteoglycans, glycosylphosphatidylinositol (GPI)-anchored proteins, chitin, glycolipids, mucins, O-linked oligosaccharides and O-linked GlcNAc (Hemming, 1974). However, there are only sparse reports on the purification of this key enzyme although there is a great diversity of this sugar.

According to Cell Signalling Technology (2012), there are two isoforms of *AGX* which are *AGX1* and *AGX2*. *AGX1* is a homodimer and has 2 to 3 times higher activity towards GlcNAc-1-P whereas *AGX2* is a monomer and has 8 times more activity towards GlcNAc-1-P. *AGX* belongs to the UDP-glucose pyrophosphorylase (UDPGP) type 1 family. There are various functions of *AGX* which acts as transferase and carbohydrate metabolism such as amino sugar and nucleotide sugar. The cellular component of *AGX* can be found in the cytoplasm, plasma membrane, nucleolus, cytosol and nucleus. The molecular functions of *AGX* involved are the UDP-N-acetylglucosamine diphosphorylase activity, transferase activity and nucleotidyltransferase activity.

According to Szumilo *et al.* (1996), N-acetylglucosamine (GlcNAc) is a type of sugar which is important in complex carbohydrates. GlcNAc is a component of N-linked oligosaccharides, O-linked oligosaccharides, and glycolipids. Hart *et al.* (1989) demonstrated that GlcNAc is linked in O-glycosidic linkage to serine and threonine residues on nuclear proteins and perhaps some other proteins. Therefore, *AGX* plays a vital role as a key enzyme in the production of GlcNAc polymers that catalysis the formation of UDP-GlcNAc via the following reaction:

$$\text{UTP} + \text{GlcNAc-1-P} \xrightarrow{AGX} \text{UDP-GlcNAc} + PP_i$$

According to the study conducted by Bulik *et al.* (2003), the addition of glucosamine (GlcN) to the growth medium of the cell wall eukaryotic organisms cause a rapid increase in chitin synthesis without any pronounced change in the expression of more than 6,000 genes monitored with Affymetrix gene expression chips. Thus, GlcN is also involved in the growth medium which leads to a three- to fourfold increase in cell wall chitin levels.